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ARTICLE PRINCIPAL

Recouvrant en partie

LA THESE DE DOCTORAT ES SCIENCES NATURELLES

présentée

A L'UNIVERSITE PARIS VI

par

Christian SALET

pour obtenir

le grade de Docteur Es-Sciences

Sujet de la Thèse: CONTRIBUTION A L'ETUDE DES EFFETS PRODUITS PAR MICRO-IRRADIATION DES MITOCHONDRIES PAR LASER

Soutenue le 16 Février 1972 devant la Commission d'Examen

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A STUDY OF BEATING FREQUENCY OF A SINGLE MYOCARDIAL CELL

I. Q-switched Laser Microirradiation of Mitochondria

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SUMMARY

Several mitochondria in a new-born rat myocardial cell were irradiated with a very short pulse of high power density light. The wavelength was 5 300 Å and the diameter of the spot, 5 μm. For a dose of 10^8 W/cm^2 delivered in 30×10^{-9} sec, the thermal coagulation of Janus green B (JGB)-stained mitochondria was obtained and the cell beating contractions were stopped. Yet by irradiating non-stained cells in the same way, the beating frequency was increased in one cell out of three. Eight accelerated cells examined under the electron microscope showed no apparent lesions. A myocardial cell population irradiated with the same power density in a flash photolysis apparatus showed, at 4 100 Å, a transient absorption followed by a continuous absorption indicating a photoreduction. A mechanism of action is suggested to explain this photoactivation.

Many studies have been made in an effort to elucidate the factors determining the beating frequency of myocardial cells in monolayer culture. The rate of beating which varies widely from cell to cell [14], and for each cell, is dependent primarily upon the temperature of the preparation [12]. When two cells beating at different rates come in contact, a synchronization of their rates of contraction occurs. This phenomenon has been extensively studied [9, 10], but less attention has been given to the regulation of the beat of an isolated cell. In the present paper, we deal with the rhythmic contraction of a single cell, physically isolated from all others, as studied by laser microirradiation.

Since heart muscle cell metabolism is primarily aerobic [15], a physical action at the level of the mitochondria is very efficient. Rounds et al. [16] were the first to report a photochemical action of green laser light on biological material as a result of absorption by cytochromes $c+c_1$. In the same way, Berns et al. [4] using a laser beam of low divergency have shown that it is possible to produce a lesion by microirradiation in one mitochondrion of a rat heart muscle cell, usually with no change in the beating frequency of the cell. We reported earlier [18] that it is possible to increase the beating frequency of about 30% of the treated cells when several mitochondria are irradiated with a very short pulse of high power density light. A complete account of these microirradiation experiments is presented here. It includes: (1) an attempt to distinguish between thermal and photochemical action at the level of irradiated mitochondria in connection with beating frequency changes; (2) electron microscopic observations of the irradiated cells; (3) the detection of photochemical reactions by flash photolysis.

MATERIALS AND METHODS

Source

We used a Q-switched neodymium laser. By doubling the frequency with a KDP crystal, we obtain 3 J at 5 300 Å. The pulse width is 30×10^{-9} sec.

Radiation equipment

This is derived from the classical ultraviolet equipment of Bessis & Nomarski [5]. The focusing of the laser beam is effected with the aid of an achromatic objective ($\times 32$, numerical aperture 0.40) without an intermediate lens. The diameter of the central zone of irradiation is about 5 μm. A continuous He-Ne laser, coaxial with the Q-switched laser is used for locating the target area and focusing prior to irradiation. The observations are made with phase contrast optics.

Measurements

Measurements of the dose of radiation were made at the input of the equipment by photoelectric cells with a rapid response. The signals furnished by these cells were systematically photographed on the screen of a fast oscilloscope. Radiation energy was varied with the aid of a combination of grey filters; the transmission of the focusing system was taken into consideration in calculating dosage. At the output of the radiation equipment, a photomultiplier detects the fluctuation of the continuous He–Ne laser through the preparation. The modulation of the flux by diffusion and diffraction of droplets moving in the target cell is sufficient to produce a modulation of the photomultiplier signal. The photomultiplier output is transferred to a chart recorder that produces a good direct record of the contraction rhythm.

Flash photolysis apparatus

The flash photolysis technique is a powerful tool for studying photochemical reactions. It involves looking at the variation in optical density of irradiated material immediately after irradiation. For obvious signal-to-noise ratio reasons, it is impossible to employ this method using just a single cell. Therefore, we used a cell population (4 millions/cm^3) with the flash apparatus described previously [2]. To avoid contamination by red cells, the myocardial cells were prepared via tissue culture. For treatment, the cells were placed in a small cuvet and irradiated with the laser beam. A simplified schematic diagram of the experimental apparatus is provided in fig. 1.

For monitoring purposes a small part of the laser beam is deflected to a fast photocell, whose output is displayed on a dual beam oscilloscope. The same oscilloscope simultaneously records, via a photomultiplier, changes produced by the laser flash in the analysing light which passes through the reaction vessel. The continuous spectrum of the analysing flash was resolved by a grating monochromator before entering the photomultiplier. The time resolution was about 5×10^{-9} sec.

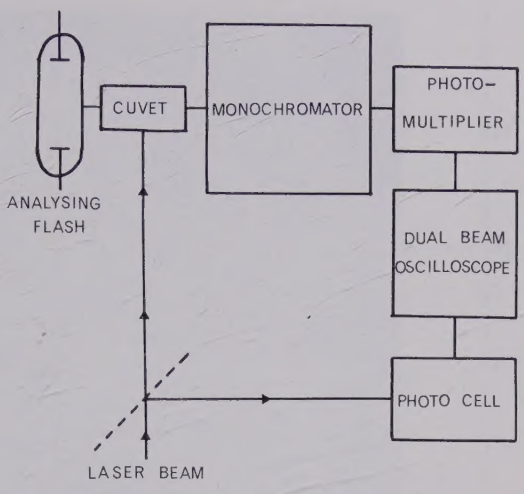

Fig. 1. Simplified diagram of the flash photolysis apparatus. See text for explanation.

Cells in culture

The myocardial cell culture was made according to the method of Mark & Strasser [14]. The heart of a new-born rat is removed and cut into small pieces. After trypsinization (trypsin 0.25 % in balanced salt solution), the cells are placed in Sykes-Moore chambers and incubated with daily medium changes. Nutrient medium [11] was supplemented with 18 % fetal bovine serum, antibiotics and vitamins. The cells were generally irradiated on the fourth day in slide–coverslip preparations at room temperature (20°C). According to Harary [12], they are able to beat several hours with a mean frequency of 20 to 25 beats/min.

For some experiments cells were stained with a specific mitochondrial dye, Janus green B (Gurr), dissolved in the culture medium [19, 21, 22]. Concentrations are expressed in g of JGB per g of solvent.

Electron microscopy

Irradiated cells were examined in a Siemens electron microscope (Elmiskop 1). The transfer from the optical microscope to the electron microscope was made using the technique described by Storb et al. [21]. The cells were fixed with 3 % glutaraldehyde in 0.1 M Sörensen buffer for 10 min, washed in the buffer, post-fixed with 1 % osmium tetroxide in the same buffer for 10 min. Staining of sections was made according to Reynolds [17].

RESULTS

Microirradiation at 5 300 Å of unstained cells

Only isolated cells were irradiated. In every case the irradiation site was located in a part

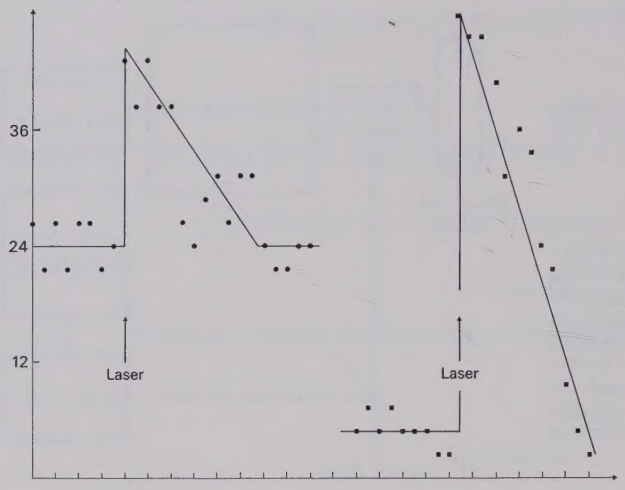

Fig. 2. Abscissa: number of beats per minute; *ordinate:* time (50 sec per division). Each point represents the frequency calculated in a 25 sec period.

of the cytoplasm very rich in mitochondria, but also likely to contain other organelles such as lysosomes, ribosomes or myofibrils.

For a flux of approx. 10^9 W/cm² or more, the cell was killed. In four experiments we studied the irradiated cell under the electron microscope following fixation within 1 min of treatment. Under these conditions the mitochondria were swollen and the matrix was somewhat less dense to the electrons. The cristae were still present but seemed less organized (fig. 3). The other organelles seemed to be morphologically normal, except for the cytoplasmic membrane which was damaged.

For a flux of about 10^8 W/cm², an acceleration of contraction was observed in one experiment out of three. Such acceleration lasts typically for approx. 3 min, then the cell returns to its former rhythm (fig. 2). We repeated the experiment several hundred times and studied eight accelerated cells by electron microscopy. No lesions were detected in such cells or in controls (fig. 4).

In some cases the irradiation of a non-beating cell started the rhythmic contraction. In addition, we have observed that it is difficult to stimulate the same cell successively (at 5 min intervals) over an extended period of time.

Microirradiation at 5 300 Å of JGB-stained cells

We stained heart muscle cells in culture medium containing JGB in concentrations of 1.0 or 2.5×10^{-6}. Under these conditions, the stained cells were able to beat between slide and coverslip for several hours. Following irradiation with doses below 10^8 W/cm², no particular effects were observed. But, on increasing the dose just above 10^8 W/cm², the beating was immediately interrupted. For a dose of 10^9 W/cm² we obtained small coagula, visible under phase contrast. This effect has been studied in detail on JGB-stained KB cells irradiated with a laser [19]. For the heart muscle cells, coagulation of the mitochondria occurs at the same dose of absorbed radiation when the dye concentration in the medium is 10^{-6} in both cases.

Transient absorption in non-stained cells irradiated with 10^8 W/cm²

As discussed below, the effect obtained without dye is not a thermal one. As far as is

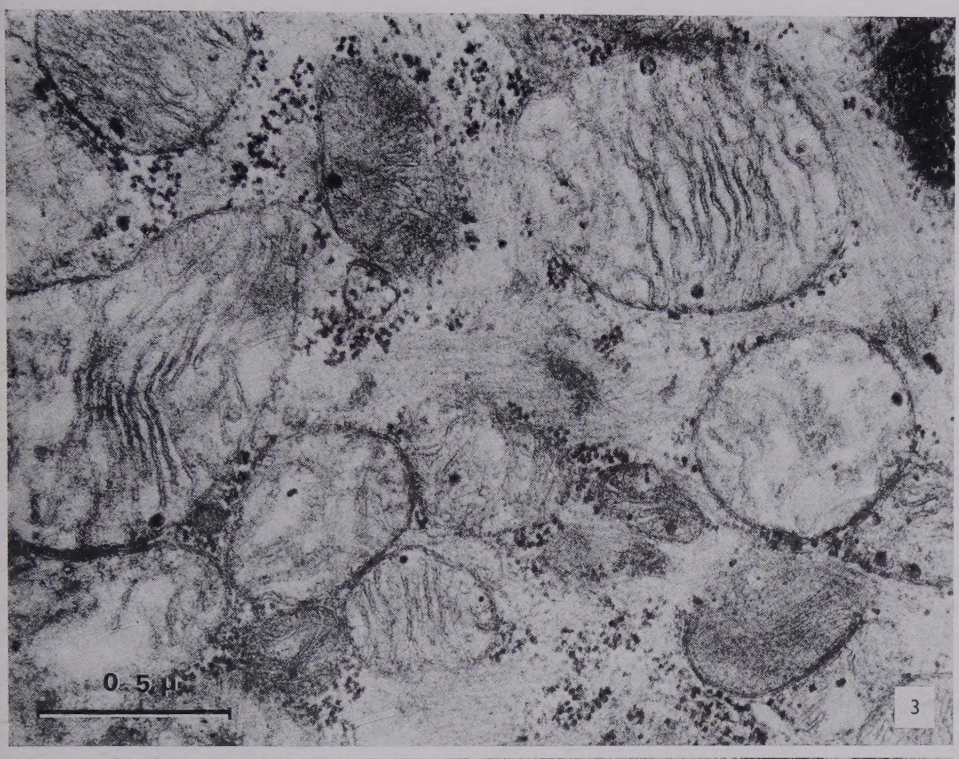

Fig. 3. Mitochondria of an unstained rat myocardial cell irradiated with 10^9 W/cm² of 5 300 Å laser light. The mitochondria are swollen and their cristae disorganized.
Fig. 4. Mitochondria of rat myocardial control cell. The cells irradiated with 10^8 W/cm² of 5 300 Å laser light look like controls, as explained in the text.

known, the only mitochondrial constituents that absorb at a wavelength of 5 300 Å are cytochromes, particularly *c* and c_1. The photochemistry of cytochrome *c* and its photoreduction in the presence of EDTA will be treated in detail elsewhere. Our only purpose here is to provide qualitative data relating to the possibility of photochemical reactions at the level of the mitochondrion. As explained earlier, for signal to noise ratio reasons, it is impossible to work with only one cell in the photolysis apparatus. Accordingly, we used a cell population in the cuvet and employed the same green laser light, without localization, for excitation purposes. The power density was 10^8 W/cm^2, i.e., the power density used to stimulate a single myocardial cell by partial cell irradiation.

When fresh cells are irradiated in the apparatus one observes at the wavelength of 4 100 Å, where the difference of absorption between the reduced and oxydized form of cytochrome *c* is maximum, a short-lived transient absorption (life time = 1 μsec) followed by a weak continuous absorption indicating a photoreduction. This change in absorption was at the limit of sensitivity of our apparatus. Therefore, the whole difference spectrum cannot be given, but it was verified that no change in absorption was detectable at the isobestic point. After several laser shots in the same cell suspension the resulting oscillogram at 4 100 Å changes progressively and resembles closely the oscillogram obtained with cells reduced with 10^{-3} M of KCN [20]. These experimental data suggest a reduction at the level of cytochrome *c*.

DISCUSSION

The selective interaction between laser light and mitochondria results from localisation of the radiation by the microbeam technique and from specific mitochondrial absorption. It is well known that, except for substances like hemoglobin and the cytochromes, mammalian cell constituents do not absorb in the visible range. The presence of cytochromes in the mitochondria gives them a weak absolute absorption, but a strong relative one compared with other organelles such as the nucleus, Golgi apparatus and so on. Such absorption can be increased by employing selective JGB staining of the mitochondria. Theoretically, in such a situation, the absorbed electromagnetic energy can be reemitted as fluorescence or phosphorescence, degraded to heat, or used in chemical modification [3]. The two last-named possibilities are particularly pertinent to our study and will now be examined.

The effects obtained by increasing the absorption of cytochromes by the addition of JGB are thermal; one obtains, in the focal volume, the coagulation of the mitochondrion by irreversible heat degradation of its constituent proteins. This change is visible in the phase microscope because it is correlated with a change in the index of refraction [1]. Because of the very short exposure time employed in our experiments, thermal diffusion during irradiation can be neglected, since the degradation is restricted to the mitochondrion. These points have been discussed in detail in a previous paper [19]. In these experiments the presence of dye could influence the biological response. However, the cells were able to beat several hours after staining. For a greater concentration (10^{-5} JGB) it is reported that the cells were able to beat for 105 min [13].

With a less divergent laser beam and a 10^3 fold longer period of illumination (ionized-argon laser $\tau_i = 50 \times 10^{-6}$ sec, $\lambda = 4\,880$ Å and 5 145 Å), Berns et al. [4] have shown that it is possible to produce a lesion limited to one mitochondrion in an unstained cell. In these experiments, the power density was

also 10^8 W/cm^2. The nature of the lesions is not described but their appearance under phase contrast suggests that a thermal effect may be involved.

It is possible to calculate the maximum temperature reached at the hottest point in an irradiated volume if the incident power density and the absorption of the target volume are known. For mitochondria it is admitted [4, 16, 23] that the cytochromes are the only intramitochondrial substances able to produce a perceptible absorption in the visible range. It is possible to evaluate mitochondrial absorption knowing the molecular concentration in mitochondria of the rat myocardial cell [24] and the extinction index of cytochrome *c*. With a mitochondrion of 1 μm diameter, the coefficient of absorption is approximately the same at both 5 300 Å and 5 145 Å and its value is 5×10^{-4}. A simple calculation [6], taking into account thermal diffusion, is possible if we know the duration of illumination, the incident power density and the coefficient of absorption of a mitochondrion. In the case of ionized-argon irradiation ($\tau_i = 50$ μsec), with a power density of 10^8 W/cm^2, the order of magnitude of the temperature increase at the hottest point is 100°C. This is another indication of a thermal effect.

Thermal diffusion is more important with a longer irradiation time (i.e. at the end of the laser heating pulse there is a sharp temperature gradient around the absorbing target with a Q-switched laser but a weak one with an ordinary laser which has a longer duration of illumination). In Berns's experiments [4] if we assume that the effect is a thermal one, the lesion is not limited as closely to the mitochondrion. This could explain the greater diversity in his biological results especially when the irradiated mitochondrion is near the nucleus which could be heated by thermal diffusion. Thus, it can be concluded that a coagulation limited to one mitochondrion, or a part of a mitochondrion, has little influence on beating frequency, but when several are irradiated in the same way the contractions are stopped.

The results obtained in non-stained cells irradiated with the Q-switched laser are difficult to explain. The results of irradiation with 10^9 W/cm^2 or above show obvious morphological changes in the cell: the mitochondria are swollen and the cytoplasmic membrane is damaged. These changes could be attributed to the beginning of dielectric breakdown. We have reported the value 10^{10} W/cm^2 as the threshold of dielectric breakdown [19], but other authors have given lower values for a liquid with particles in suspension [8]. The effects induced by irradiation with 10^8 W/cm^2 could not be of thermal origin for several reasons. The calculated value of the greatest possible temperature change is small (1°–4°C). Besides, comparison with previous experiments is perfectly compatible with this evaluation. If we assume that the maximum temperature jump is 100°C when a mitochondrion is irradiated with the ionized-argon laser ($\tau_i = 50 \times 10^{-6}$ sec, power density 10^8 W/cm^2) the change in temperature must be very small when the duration of the illumination is only 30×10^{-9} sec (Q-switched-laser $\lambda = 5\,300$ Å, the absorption of the cytochromes is of the same order of magnitude at this wavelength as it is at the argon laser wavelengths). The experiments involving the addition of dye to the mitochondrion prove also a contrario that the effect cannot be a thermal one. These facts are confirmed by the appearance of the irradiated cells in electron microscopy. When a part of a cell is thermally denatured, it has been shown that the denatured portion, after classical fixation and staining, is denser to electrons [19, 21]. In the present case, no electron-dense thermal lesion appears.

The cytoplasmic membrane appears to be

morphologically the same as in control cells. A lesion in this structure could lead to a change in the normal ion density on each side of the membrane and thus perturb the intracellular potential which is related to the beating frequency. We have not actually made measurements of the action potential. However, since the membrane is unable to absorb in the visible range and shows no thermal lesion, there is a low probability that such a mechanism is implicated in beating frequency changes.

If we disregard thermal effects as a causative agent, it is possible that the observed effect is a photochemical one produced by specific absorption of the mitochondrial pigments. In fact the flash photolysis experiments provide strong evidence for that possibility. The presence of myoglobin in the extra-mitochondrial parts of the cells could however, perturb the interpretation of the transient change absorption. But, if the cytochromes are really reduced and if we consider that the reactions in the oxidation-reduction chain of the mitochondria are reversible [7], we conclude that the free energy of the mitochondrion is increased. We suggest as an hypothesis that this supplementary energy could be used for increasing ATP phosphorylation. The result could be an augmentation of the beating frequency as has been suggested by Yaffe [25]. If this hypothesis is true, the heart muscle cell is apparently able to convert, with a low quantum yield,electromagnetic energy into mechanical energy.

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